***Pimephales promelas* 7-Day Chronic Toxicity Test**

1.0 OBJECTIVE

In laboratory tests designed to determine the toxicity of low salinity water samples, fathead minnow juveniles are exposed to test solutions for 7 days, after which the survival and growth are determined in each toxicant concentration or sample. Observed effects may be related to the presence of contaminants or to naturally occurring factors. In order to correctly interpret toxicity results, concentrations of chemical contaminants should be analyzed, as well as other water quality parameters, such as pH, dissolved oxygen, total dissolved solids, hardness, alkalinity, temperature, ammonia and conductivity.

In this procedure, water samples collected from field stations are divided into replicate beakers in the laboratory. Ten fathead minnow juveniles are placed into each replicate container and monitored for mortality and growth. After a 7-day exposure, daily survival and final growth are used to give an estimate of sample toxicity. Because the test measures effects on an early life-stage of an ecologically important species possessing relatively stringent water quality requirements, the results constitute a good basis for decisions concerning either hazard evaluation or the suitability of estuarine waters for aquatic life (US EPA 1994).

2.0 EQUIPMENT

The following equipment is necessary to conduct the toxicity test at the Marine Pollution Studies Laboratory at Granite Canyon (MPSL). The word "clean" here and throughout this procedure means that the item has been cleaned according to the MPSL glassware cleaning procedures outlined in a separate standard operating procedure (MPSL SOP 1.3).

2.1 Culture

- Air stones and clean air system
- 4:1 water prepared from Perrier® or Evian® and distilled water (25 ± 1°C.)
- Newly hatched *Artemia* for feeding
- Disposable plastic pipettes (for handling fish)

2.2 Test Initiation

- 500-mL glass beakers (4 per sample or concentration)
- 1000-mL volumetric flasks and pipettes for reference toxicant dilutions
- 1000-mL plastic tri-pour beakers
- Water bath or environmental chamber
- Data sheets
- Gloves and appropriate safety gear (see MPSL lab safety manual)
- Sample vials for reference toxicant analysis (new polyethylene 60 mL)
- Graduated pipettes

• Analytical balance

• Plastic squirt bottles

2.3 Water Quality

• Meters and probes for measuring pH, dissolved oxygen, hardness, alkalinity, ammonia, and conductivity

• Thermometers (glass spirit thermometer and continuously recording thermometer)

• Graduated pipettes and hand pipette pump for water quality sampling

• Gloves and appropriate safety gear (see MPSL lab safety manual)

2.4 Dilution Water

In every step of this procedure, use Granite Canyon Nanopure® water mixed with Evian® in a 4:1 ratio. Conductivity should not exceed 3000 µS/cm. Hardness (as $CaCO_3$) should not exceed 700 mg/L.

3.0 EXPERIMENTAL DESIGN

Aquatic toxicity tests can be used as screening tools or as part of more comprehensive studies to assess water quality. Careful consideration must be given to site characteristics, reference site selection, field replication, choice of synoptic measures, seasonal factors, and comprehensive planning and peer review to determine that study designs are adequate to meet program objectives.

This laboratory toxicity test consists of four replicate test containers for each sample concentration. The quality of test animals and testing conditions is determined through concurrent testing of reference toxicants (positive controls) and control water (negative controls). Testing of reference sites or receiving water is recommended to demonstrate the suitability of test sites in the absence of toxic contaminant concentrations. Dissolved oxygen, pH, conductivity, ammonia, alkalinity, and hardness are measured at the beginning of the exposure. New and old dissolved oxygen and old pH are measured daily. Temperature is measured daily by hand and measured continuously by a temperature logger. The photoperiod for the test is 16 hours light: 8 hours dark, and the temperature is 25 ± 1°C.

4.0 SAMPLE PREPARATION

Because of the 48-hour holding time, tests will generally be initiated on the same day as sample receipt. Filter sample through a 25-µm screen and place appropriate sample volume in the constant temperature room. Allow oxygen concentrations to equilibrate below super-saturated levels. Aerate if super saturated.

5.0 CONTROLS

5.1 Dilution Control

The dilution control should consist of 4:1 culture water.

5.2 Reference Toxicant Test

A reference toxicant test must be conducted monthly to indicate the sensitivity of the organisms and the suitability of the test methodology. Reagent grade copper chloride ($CuCl_2$) should be used as the reference toxicant for fathead minnow tests, unless another toxicant is specified by the Regional Water Quality Control Board or other appropriate regulatory agency. Prepare a 10,000 µg/L $CuCl_2$ stock solution by adding 0.0268g reagent grade $CuCl_2$ to one liter of Nanopure water in a volumetric flask. Cap tightly and mix thoroughly. Sample the reference toxicant stock solution at the beginning of the test for chemical verification of the copper concentration. Acidify samples for analysis in clean sample vials with 1% by volume 14N reagent grade nitric acid.

Reference toxicant solutions should be four replicates of 0, 18, 32, 56, 100, and 180 µg/L. Other concentrations may be added between these if greater precision is desired for quality control chart purposes. Prepare concentrations according to dilution schedule. Start with the control solutions and progress to the highest concentration to minimize contamination. Use plastic volumetric flasks and beakers to reduce loss of copper to container walls.

All tests (sample and reference toxicant) must use juveniles from the same culture. They must be handled in the same way and delivered to the test containers at the same time.

6.0 TEST INITIATION

Measure temperature, dissolved oxygen, pH, conductivity, ammonia, alkalinity, and hardness. Fathead minnow juveniles are randomly placed in all test containers until each replicate contains ten fish. Test containers should be randomized according to the test set-up sheet.

7.0 DAILY TEST MONITORING AND RENEWAL

7.1 Feeding

Feed each replicate twice per day. Rinse newly hatched *Artemia* nauplii with fresh water to removes salts. One mL of rinsed and concentrated nauplii is approximately equivalent to one gram. Pipette 5 mL of concentrated nauplii into a small flask or beaker and add 10 mL control water. Feed each replicate 2 drops (0.15g) in the morning, and at least 6 hours later. On weekend days, replicates should be fed at the beginning and end of the work shift. If survival rate in any test container falls below 50%, reduce feeding to one drop.

7.2 Counting and Renewal

Count the number of surviving fish daily. As counts are being made, use a disposable transfer pipette to remove dead *Artemia* and dead fish. After morning feeding and count, renew the test solutions. Using a siphon (glass tube attached to plastic tubing), remove 80% of the test solution by siphoning into a beaker. Fish that are accidentally siphoned can be returned to the test beaker. Slowly pour new sample water down the side of the test containers to minimize stress to fish. Measure temperature, new dissolved oxygen and old dissolved oxygen and pH.

8.0 TERMINATING THE TOXICITY TEST

After 7 days of exposure final survival counts are made. Measure temperature, dissolved oxygen and pH. Pour contents of beaker through a 500µm screen, rinse fish with Nanopure water, and transfer to pre-weighed pan for weight measurements. Dry the fish overnight in an oven set to 60°C. After 24 hours place weigh pans in a desiccator to cool. Measure final weight to the nearest 0.01 mg. Take the completed data sheet to the office for data entry and analysis. Notify the data analyst that the data has arrived.

9.0 DATA HANDLING AND TEST ACCEPTABILITY

Immediately after test termination, check the data sheet to determine whether control has acceptable survival. This toxicity test procedure is considered acceptable if fathead minnow survival in controls is greater than or equal to 80%. Average dry weight of surviving fish in controls must exceed 0.25mg. Tests with temperature, salinity, or dissolved oxygen measurements outside the specified ranges, may be considered conditionally acceptable based on the project officer's best professional judgment. Acceptable temperatures range from 25 ± 1°C; acceptable dissolved oxygen concentration is 4.0 mg/L to 110 percent saturation.

10.0 REFERENCES

U.S. Environmental Protection Agency. 2002. Short-term methods for estimating the chromic toxicity of effluents and receiving water to freshwater organisms. EPA-821-R-02-013. Office of Research and Development. Washington, DC.

11.0 TEST SUMMARY

Species:	*Pimephales promelas*
Test Duration:	7 days
Renewals:	Daily
Organism Source	Aquatic Biosystems
Age of test organisms:	<24 hours
Temperature:	25 ± 1°C recommended (range must not exceed 3°C required)
Dissolved Oxygen	>4 mg/L recommended

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Light intensity:	Ambient laboratory illumination 10-20 µE/µ^2/s
Photoperiod:	16 hour Light: 8 hour Dark; Replication: 5 replicates
Test Containers:	500-mL beakers
Test solution volume:	250-mL
Loading:	10 fish per beaker
Feeding:	Newly hatched, rinsed *Artemia*
Water Quality:	Dissolved oxygen, pH, conductivity, ammonia, alkalinity, hardness, and temperature
Reference Toxicant:	Copper Chloride ($CuCl_2$)
Stock Solution:	0.0268 g in 1 liter of distilled water (10,000 mg/L).
Dilutions:	0, 18, 32, 56, 100, 180 mg/L
Daily Monitoring:	Survival
Safety:	Wear protective clothing; read applicable MSDS, be familiar with the lab safety manual prior to testing.
Quality Control:	Fill out all data sheets completely. Be familiar with QA Project Plan prior to testing.
Acceptability Criteria:	Dilution Controls ≥80% Average dry weight of control fish >0.25mg
Temperature range:	24° to 26°C.